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Process for Preparing Doxorubicin.

Field of the Invention

The present invention concerns a process for improving daunorubicin to doxorubicin conversion by means of host cells transformed with recombinant vectors comprising DNA encoding a daunorubicin C-14 hydroxylase together with genes conferring resistance to anthracycline antibiotics.

Background of the Invention

Anthracyclines of daunorubicin group such as doxorubicin, carminomycin and aclacinomycin and their synthetic analogs are among the most widely employed agents in antitumoral therapy (F. Arcamone, Doxorubicin, Academic Press New York, 1981, pp. 12; A. Grein, Process Biochem., 16:34, 1981; T. Kaneko, Chimicaoggi May 11, 1988; C. E. Myers et al., "Biochemical mechanism of tumor cell kill" in Anthracycline and Anthracenedione-Based Anti-cancer Agents (Lown, J. W., ed.) Elsevier Amsterdam, pp. 527-569, 1988; J. W. Lown, Pharmac. Ther. 60:185, 1993).

Anthracyclines of the daunorubicin group are naturally occurring compounds produced by various strains of *Streptomyces* (*S.peucetius*, *S.coeruleorubidus*, *S.galilaeus*, *S.griseus*, *S.griseoruber*, *S.insignis*, *S.viridochromogenes*, *S.bifurcus* and *S.sp.* strain *C5*) and by *Actinomyces carminata.* Doxorubicin is mainly produced by strains of *S. peucetius*. In particular daunorubicin and doxorubicin are synthesized in *Streptomyces peucetius* ATCC 29050 and in *S. peucetius subsp. caesius* ATCC 27952. The anthracycline doxorubicin is made by *S.peucetius* 27952 from malonic acid, propionic acid and glucose by the pathway summarized in Grein, Advan. Applied Microbiol. 32:203, 1987 and in Eckart and Wagner, J. Basic Microbiol. 28:137, 1988. Aklavinone (11-deoxy-ε-rhodomycinone), ε-rhodomycinone, rhodomycin D, carminomycin and daunorubicin are established intermediates in this process. The final step in this pathway involves the C-14 hydroxylation of daunorubicin to doxorubicin.

Genes for daunorubicin biosynthesis have been obtained from *S.peucetius* 29050 and *S.peucetius* 27952 by cloning experiments (Stutzman-Engwall and Hutchinson, Proc.Natl.Acad.Sci.USA 86:3135,1988; Otten et al., J.Bacteriol. 172:3427,1990).The gene encoding the daunorubicin 14-hydroxylase, which converts daunorubicin to doxorubicin has been obtained from *S.peucetius* 29050 and its mutants by cloning experiments and it was overexpressed in the host cells of *Streptomyces* species and *Escherichia coli* as described in WO 96/27014, publication date

Sept.6,1996.

Two genes of the daunorubicin biosynthetic cluster, *drrA* and *drrB*, which confer doxorubicin and daunorubicin resistance to *Streptomyces lividans* have been cloned from *S. peucetius* ATCC 29050 strain (Guilfoile and Hutchinson, Proc.Natl.Acad.Sci.USA 88:8553, 1991) (Accession Number M73758 of Genbank) and from the *S.peucetius* 7600 mutant (EP-0371,112-A and Colombo et al., J.Bacteriol.174:1641,1992). These genes encode two translationally coupled proteins, both of which are required for daunorubicin and doxorubicin resistance in this host. The sequence of the predicted product of one of the two genes is similar to the products of other transport and resistance genes, most notably the P-glycoproteins from mammalian tumor cells. Another gene, *drrC,* which confers resistance to daunorubicin and doxorubicin with a strong sequence similarity to the *Escherichia coli* and *Micrococcus luteus* UvrA proteins involved in excision repair of DNA has been cloned from *S.peucetius* ATCC 29050 (Lomovskaya et al., J.Bacteriol.178:3238, 1996).

Summary of the invention

The present invention provides a process for improving daunorubicin to doxorubicin conversion in host cells by means of recombinant vectors comprising a DNA region or fragment containing the gene *dxrA* encoding daunorubicin 14-hydroxylase together with a DNA region or fragment containing one, two or three genes, selected from the group consisting of *drrA, drrB* and *drrC*, conferring resistance to daunorubicin and doxorubicin. The last three genes confer a high level of resistance in the host cells to doxorubicin, the product of the conversion process, making the process more efficient than the previous one obtained using host cells transformed with the recombinant vectors carrying only the DNA fragment containing the *dxrA* gene, described in WO 96/27014, even when a strong promoter is used.

The DNA of the invention comprises preferably all three of the *drrA, drrB* and *drrC* genes or only the two *drrA* and *drrB* genes.

The DNA may be ligated to a heterologous transcriptional control sequence in the correct fashion or cloned into a vector at the restriction site appropriately located

near a transcriptional control sequence in a vector. Typically, the vector is a plasmid. The recombinant vectors may be used to transform a suitable host cell. The host may be strains of Actinomycetes that do not or do produce anthracyclines, preferably strains of *Streptomyces* .

Brief description of the drawings

Fig. 1 (a-c) illustrate the construction of the plasmid pIS156 described in Example 1. This plasmid was constructed by insertion of the 2.9 kb fragment containing the *doxA* (formerly *dxrA*), the *dnrV* (formerly *dnrORF10*) and the C-terminal part of the *dnrU (ΔdnrU,* formerly *dnrORF9)* genes, obtained from the recombinant plasmid pIS70 (WO 96/27014 and A. Inventi Solari et al., GMBIM '96, P58), under the control of the strong promoter *ermE** (Bibb et al., Molec. Microbiol. 14:533, 1994) into the plasmid pWHM3 (Vara et al., J. Bacteriol. 171:5872, 1989).

In order to better describe the invention, we provide the SEQ.ID. No:1 of 2.867 nt consisting of the *doxA, dnrV* and the C-terminal part of the *dnrU (ΔdnrU)* genes (complementary strand to the coding strand).

Fig. 2 (a-d) illustrate the construction of the plasmid pIS284 described in Example 1. This plasmid contains the 2.9 kb fragment encompassing the *doxA*, the *dnrV* and the C-terminal part of the *dnrU* genes, obtained from the recombinant plasmid pIS70, under the control of the strong promoter *ermE** together with a DNA fragment of 2.3 Kb including the *drrA* and *drrB* resistance genes obtained from the plasmid pWHM603 (P. Guilfoile and C.R. Hutchinson, Proc. Natl. Acad. Sci. USA 88:8553, 1991) subcloned into the plasmid pWHM3.

Fig. 3 (a-c) illustrate the construction of the plasmid pIS287 described in Example 2. Said plasmid was constructed by insertion of the 2.9 kb *BamHI-HindIII* fragment containing the *doxA* formerly, *dxrA), dnrV* (formerly *dnr-ORF10)* and the C-terminal part of the *dnrU (ΔdnrU,* formerly, *dnr-ORF9)* genes, obtained from the recombinant plasmid pIS70 (WO 96/727014), under the control of the strong promoter *ermE** together with the 2.3 kb *XbaI-HindIII* DNA fragment containing the *drrA* and *drrB*

resistance genes and the 3.9 kb *EcoRI-HindIII* fragment containing the *drrC* resistance gene into the plasmid pWHM3.

The maps shown in Figs. 1,2 and 3 do not necessarily provide an exhaustive listing of all restriction sites present in the DNA fragments. However, the reported sites are sufficient for an unambiguous recognition of the DNA segments.

Restriction sites abbreviations: *Ap*, apramycin;*tsr*, thiostrepton, *amp*, ampicillin; B, *Bam*HI; G, *Bgl*II; *N*, *Not*I; *K*, *Kpn*I; *E*, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sph*I; X, *Xba*I, L, *Bgl*I; T, *Sst*I.

Detailed description of the invention.

The present invention provides a DNA molecule in which a DNA region or fragment containing the gene encoding a daunorubicin C-14 hydroxylase is joined to a DNA region or fragment containing one, two or three different genes selected from the group consisting of *drrA, drrB, drrC* genes encoding proteins conferring to the host cells resistance to daunorubicin and doxorubicin.

The DNA region containing the gene encoding a daunorubicin C-14 hydroxylase is preferably the 2.9 kb DNA region obtained from the recombinant plasmid pIS70 described in the patent WO 96/27014 by digestion with *Bam*HI-*Hin*dIII enzymes. This fragment contains the *doxA* gene, encoding the C-14 hydroxylase. Daunorubicin C-14 hydroxylase converts daunorubicin to doxorubicin. The 2.9 kb DNA fragment also comprises the *dnrV* gene between the *Not*I-*Kpn*I sites and a *Not*I-*Sph*I fragment containing the C-terminal part of the *dnrU* (ΔdnrU) gene.

Preferably, this 2.9 kb DNA fragment encoding a daunorubicin C-14 hydroxylase was ligated to both the 2.3 kb *Xba*I-*Hin*dIII DNA fragment containing the *drrA* and *drrB* resistance genes obtained from the plasmid pWHM603 and the 3.9 kb *Eco*RI-*Hin*dIII fragment containing the *drrC* gene obtained from the plasmid pWHM264; in another preferred embodiment, the 2.9 kb DNA fragment is ligated to the 2.3 kb *Xba*I - *Hin*dIII DNA fragment only.

All the DNA molecules encoding a daunorubicin C-14 hydroxylase described in WO 96/27014 may be employed in the present invention.

In particular the DNA molecule of the present invention may comprise all of the 2.9 kb DNA fragment or only a part of the fragment, at least 1.2 kb in length corresponding to the *Kpn*I-*Bam*HI fragment containing the DNA molecule of *doxA*, encoding a daunorubicin C-14 hydroxylase, which converts daunorubicin to doxorubicin. This DNA molecule consists essentially of the sequence reported in the patent application W0 96/27014, which sequence is referred to as the "*dxrA*" sequence. Also, the deduced amino acid sequence of the daunorubicin C-14 hydroxylase is shown in that patent application.

The DNA molecule of the present invention may comprise at least 2247 nt of the 2.3 kb *Xba*I-*Hin*dIII DNA fragment containing the *drrA* and *drrB* genes encoding proteins conferring to host cells resistance to daunorubicin and doxorubicin.

The DNA molecule of the invention may comprise all or part of the 3.9 kb *Eco*RI-*Hin*dIII fragment containing the *drrC* resistance gene, at least 2.5 kb in length corresponding to the *Sst*I-*Sph*I fragment containing the DNA molecule of *drrC,* encoding a protein conferring to host cells resistance to daunorubicin and doxorubicin.

The present invention also includes DNA comprising genes conferring resistance to doxorubicin and daunorubicin having a sequence at least 80% identical to the sequences of the *drrA* and *drrB* genes (Guilfoile and Hutchinson, Proc.Natl.Acad.Sci.USA 88:8553, 1991) and or *drrC* gene (Lomovskaya et al., J.Bacteriol.178:3238, 1996).

The DNA molecule of the invention may be ligated to a heterologous transcriptional control sequence in the correct fashion or cloned into a vector at a restriction site appropriately located near a transcriptional control sequence in the vector. Preferably the transcription of the different genes may be coordinated by a common strong promoter such as *ermE**(Bibb et al., Molec. Microbiol. 14:533, 1994).

The DNA molecule of the invention may be ligated into any autonomously replicating and/or integrating agent comprising a DNA molecule to which one or more additional DNA segments can be added. Typically, however, the vector is a plasmid. A

preferred plasmid is the high-copy number plasmid pWHM3 or pIJ702 (Katz et al., J. Gen. Microbiol. 129:2703, 1983). Other suitable plasmids are pIJ680 (Hopwood et al., Genetic Manipulation of *Streptomyces*. A laboratory Manual, John Innes Foundation, Norwich, UK,1985) and pWHM601 (Guilfoile and Hutchinson, Proc. Natl. Acad. Sci. USA 88:8553, 1991).

Any suitable technique may be used to insert the DNA into the vector. Insertion can be achieved by ligating the DNA into a linearized vector at an appropriate restriction site. For this, direct combination of sticky or blunt ends, homopolymer tailing, or the use of a linker or adapter molecule may be employed.

The recombinant vector may be used to transform a suitable host cells that do not or do produce anthracyclines.

The host cells may be ones that are daunorubicin or doxorubicin sensitive, i.e., cannot grow in the presence of a certain amount of daunorubicin or doxorubicin, or that are daunorubicin or doxorubicin resistant. In any case the resulting recombinant clones obtained by transformation with the new recombinant vectors of the invention show higher level of resistance to daunorubicin and doxorubicin than the parental host. The level of doxorubicin resistance in recombinant *S. lividans* is much higher than the level observed in anthracycline producing strains *S. peucetius* ATCC 29050 and ATCC 27952.

The host may be a microorganism such as a bacterium. Strains of Actinomycetes, in particular strains of *S. lividans* and other strains of *Streptomyces* species that do not produce anthracyclines may be transformed. *S. lividans* TK 23 is a more suitable host in comparison to the *S. peucetius dnrN* mutant transformed with the recombinant plasmid pIS70 containing the *dxrA* gene used for daunorubicin to doxorubicin bioconversion (WO 96/27014).

The recombinant vectors of the invention may also be used to transform a suitable host cell which produces daunorubicin, in order to enhance the conversion of daunorubicin to doxorubicin.

S. peucetius ATCC 29050 and ATCC27952 strains including their mutants that produce

anthracyclines may therefore be transformed. In particular *S. peucetius* strain WMH1654, a mutant strain obtained from *S.peucetius* ATCC 29050 and deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA, under the accession number ATCC55936 may be used.

Transformants of *Streptomyces* strains are typically obtained by protoplast transformation.

The invention includes processes for improving doxorubicin production by conversion of daunorubicin, which processes comprise a bioconversion process of added daunorubicin into doxorubicin in hosts which do not produce anthracyclines and a fermentation process for producing doxorubicin in hosts which directly produce daunorubicin.

<u>Bioconversion process of daunorubicin to doxorubicin.</u>

This process comprises:

1) culturing the recombinant host cells not producing daunorubicin transformed with the vectors of the invention to which daunorubicin is added and

2) isolating doxorubicin from the culture.

In this process the recombinant strain may be cultured at temperatures from 20°C to 40°C, for example from 24°C to 37°C. The daunorubicin is added to the culture medium from 24 to 96 hours of the growth phase. The culture is preferably carried out with shaking. The duration of the culture in the presence of daunorubicin may be from 12 to 72 hours. The concentration of daunorubicin in the culture may be from 20 to 1000 mcg/ml; for example from 100 to 400 mcg/ml.

<u>Doxorubicin production by fermentation.</u>

This process comprises:

1) culturing recombinant daunorubicin-producing host cells transformed with the vectors of the invention and

2) isolating doxorubicin from the culture.

In this process the recombinant strain may be cultured at temperature from 20°C

to 40°C; for example from 26°C to 34°C. The culture is carried out with shaking. The duration of the culture may be from 72 to 168 hours.

Materials and Methods

Bacterial strains and plasmids: *E. coli* strain DH5α, which is sensitive to ampicillin and apramycin is used for subcloning DNA fragments. The host *S. lividans* TK23 was obtained from D. A. Hopwood (John Innes Institute, Norwich,United Kingdom) and the host *S. peucetius* WMH1654 is a mutant strain obtained from *S.peucetius* ATCC 29050 and has been deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA, under the accession number ATCC55936.

The plasmid cloning vectors are pGem-7Zf(+) and related plasmids (Promega, Madison, WI), pIJ4070 (D. A. Hopwood) and the *E.coli-Streptomyces* shuttle vector pWHM3 (Vara et al., J. Bacteriol. 171:5872, 1989).

Media and buffer: *E. coli* strain DH5α is maintained on LB agar (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). When selecting for transformants, ampicillin or apramycin are added at concentrations of 100 micrograms/ml.

S. lividans TK23 and *S. peucetius* WMH1654 are maintained on R2YE (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and ISP4 (Difco, Detroit, MI) agar media, respectively. When selecting for transformants, the plates are overlayed with soft agar containing thiostrepton at a concentration of 50 micrograms/ml.

Subcloning DNA fragments: DNA samples are digested with appropriate restriction enzymes and separated on agarose gels by standard methods (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). Agarose slices containing DNA fragments of interest are

excised from a gel and the DNA is isolated from these slices using the GENECLEAN device (Bio101, La Jolla, CA) or an equivalent. The isolated DNA fragments are subcloned using standard techniques (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) into *E. coli* for routine manipulations, and *E. coli-Streptomyces* shuttle vectors or *Streptomyces* vectors for expression experiments.

Transformation of *Streptomyces* species and *E. coli*: Competent cells of *E. coli* are prepared by the calcium chloride method (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) and transformed by standard techniques (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). *S. lividans* TK23 is grown in liquid R2YE medium (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and harvested after 48 hr. The mycelial pellet is washed twice with 10.3% (wt/vol) sucrose solution and used to prepare protoplasts according to the method outlined in the Hopwood manual (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985). The protoplast pellet is suspended in about 300 microlitres of P buffer (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and 50 microlitres aliquot of this suspension is used for each transformation. Protoplasts are transformed with plasmid DNA according to the small scale transformation method of Hopwood et al. (*Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985), Stutzman-Engwall and Hutchinson (Proc. Natl. Acad. Sci. USA. 86:3135, 1988) or Otten et al. (J. Bacteriol. 172: 3427, 1990). After 17 hr of regeneration on R2YE medium at 30°C, the plates are overlayed with 200 micrograms/ml of thiostrepton and allowed to grow at 30°C until sporulated.

Evaluation of daunorubicin and doxorubicin resistance level: The level of resistance is expressed as Minimal Inhibitory Concentration (MIC) and is determined by the standard two-fold dilution method using R2YE medium. The strains are cultured in slants of R2YE medium and incubated at 28°C for 8-10 days. Recombinant strains are grown in the same medium added with 20 micrograms/ml of thiostrepton. Bacterial cultures containing approximately 10^6-10^7 viable cells/ml are prepared from cultures grown at 28°C at 280 rpm for 48 hours in Tryptic Soy Broth (Difco). The cultures are homogenized by glass beads. One loopful of the homogenized cultures is inoculated on the agar plates containing different concentrations of daunorubicin and doxorubicin from 0.39 to 800 micrograms/ml. The agar plates are incubated at 30°C for 7 days and the MICs are determined as the lowest concentrations that prevent visible growth.

Daunorubicin to Doxorubicin bioconversion: *S. lividans* TK23 transformants harboring a plasmid of the invention are inoculated into 25 ml of liquid R2YE medium with 40 micrograms/ml of thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks and incubated on a rotary shaker at 280 rpm at 30 C°. After 2 days of growth, 2.5 ml of this culture are transferred to 25 ml of APM production medium: ((g/l) glucose (60), yeast extract (8), malt extract (20), NaCl (2), 3-(morpholino)propanesulfonic acid (MOPS sodium salt) (15), $MgSO_4 .7H_2O$ (0.2), $FeSO_4 .7H_2O$ (0.01), $ZnSO_4.7H_2O$ (0.01), supplemented with 20 micrograms/ml of thiostrepton. 400 micrograms/ml of daunorubicin are added at 48 hr.of the growth phase. Cultures are grown in 300 ml Erlenmeyer flasks and incubated on a rotary shaker at 280 rpm at 30 C° for 72 hr. Each culture is acidified with 25 milligrams/ml of oxalic acid and after incubation at 30°C on a rotary shaker at 280 rpm for 30 min. is extracted with an equal volume of acetonitrile:methanol (1:1) at 30°C and 300 rpm for 2 hr. The extract is filtered and the filtrate is analyzed by reversed-phase high pressure liquid chromatography (RP-HPLC). RP-HPLC is performed by using a Vydac C_{18} column (4.6 x 250 millimeters; 5

micrometers particle size) at a flow rate of 0.385 ml/min. Mobile phase A is 0.2% trifluoroacetic acid (TFA, from Pierce Chemical Co.) in H_2O and mobile phase B is 0.078% TFA in acetonitrile (from J.T.Baker Chemical Co.). Elution is performed with a linear gradient from 20 to 60% phase B in phase A in 33 minutes and monitored with a diode array detector set at 488 nm (bandwidth 12 micrometers). Daunorubicin and doxorubicin (10 micrograms/ml in methanol) are used as external standards to quantitate the amount of these metabolites isolated from the cultures.

Doxorubicin production: The *S. peucetius* WMH1654 mutant is transformed with a plasmid of the invention. Transformants are inoculated into 25 ml of R2YE medium supplemented with 20 micrograms/ml thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks on a rotary shaker at 280 rpm at 30°C. After 2 days of growth, 2.5 ml of this culture are transferred to 25 ml of APM medium supplemented with 20 micrograms/ml thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks on a rotary shaker at 280 rpm at 28°C for 96 - 120 hours. Each culture is acidified with 25 milligrams/ml of oxalic acid and, after 45 min. incubation at 30°C on a rotary shaker at 280 rpm, is extracted with an equal volume of acetonitrile:methanol (1:1) at 30°C and 300 rpm for 2 hr. The extract is filtered and the filtrate is analyzed by RP-HPLC following the same method used to analyze the bioconversion products.

Example 1

Example 1 (Fig. 1 (a-c) and Fig. 2 (a-d).

In order to remove a non-essential region, the plasmid pIS70 (WO96/27014) is before digested *EcoRI-HindIII* and the 3.5 kb fragment is subcloned into the same sites of the multiple cloning site sequence of the plasmid pGEM-7Zf (+) (Promega, Madison-WI USA) to obtain another *BamHI* restriction site. The new plasmid pGendoxAUV was *BamHI* digested and the fragment, now reduced to 2.9 kb, was transferred into the

plasmid pIJ4070 (from the John Innes Institute, Norwich, UK) under the control of strong promoter *ermE**. This new plasmid, named p7doxAUV, was digested *BglII* and the fragment inserted into the plasmid pWHM3 (J.Vara et al., J. Bacteriol. 171:5872-5881, 1989) to obtain the plasmid pIS156 (fig. 1c).

The 2.3 kb *BglI* fragment containing the *drrA* and *drrB* resistance genes is transferred after blunt ending from the plasmid pWHM603 into the *SmaI* site of the plasmid pBluescript II SK + (Stratagene) to obtain the plasmid pdrrAB and an *XbaI-HindIII* fragment is transferred from pdrrAB into the vector pIJ4070 to obtain pIS278. Afterwards, pIS278 is digested with *EcoRI-XbaI* and inserted into the *EcoRI-XbaI* plasmid pWHM3 to obtain the plasmid pIS281. This plasmid is digested with *XbaI* and the *XbaI* fragment of plasmid pIS156 is inserted to obtain the plasmid pIS284.

Example 2

Construction of the plasmid pIS287 (Fig.3 (a-c)): The *drrC* resistance gene contained in the plasmid pWHM264 is excised by *Eco*RI-*Hin*dIII digestion and inserted into the plasmid pIJ4070 to obtain the plasmid pIS282. From this plasmid, the *drrC* resistance gene is transferred as a *Bgl*II fragment to pIS252 (this plasmid is a modified form of pWHM3 containing an extra *Bgl*II site close to the *Eco*RI site) to obtain the plasmid pIS285. pIS285 is *Eco*RI digested and ligated with the 5.5 kb DNA fragment excised from plasmid pIS284 to obtain the plasmid pIS287.

Example 3

Resistance of the above recombinant plasmids to doxorubicin: The level of resistance to daunorubicin and doxorubicin of *S. lividans* TK23 transformed with the recombinant plasmids pIS70, pIS284 or pIS287 in comparison with *S. lividans* TK23, *S. lividans* TK23 transformed with the vector pWHM3 and the anthracycline producing *S. peucetius* ATCC 29050 and ATCC 27952 strains is determined as MICs on R2YE

medium following the procedure described in Materials and Methods. The maximum level of daunorubicin and doxorubicin resistance is obtained with the plasmid pIS287 containing the *drrA*, *drrB* and *drrC* resistance genes. The level of doxorubicin resistance was increased 64 times also with the plasmid containing only the *drrA* and *drrB*.resistance genes (Table 1).

Table 1. Resistance of recombinant strains to doxorubicin.

Strain	MIC for doxorubicin (micrograms/ml)
S. peucetius ATCC 29050	12.5
S. peucetius ATCC 27952	12.5
S. lividans TK23	12.5
S. lividans TK23(pWHM3)	12.5
S. lividans TK23(pIS284)	800
S. lividans TK23(pIS287)	>800

Example 4

Bioconversion of added daunorubicin to doxorubicin in *S. lividans* TK23 transformed with plasmids containing the *doxA* daunorubicin C-14 hydroxylase gene together with different resistance genes: The pIS70, pIS284 or pIS287 plasmids are introduced into *S. lividans* TK23 by transformation with selection for thiostrepton resistance, according to the procedures described in the Materials and Methods section. The resulting *S. lividans* TK23(pIS70), *S. lividans* TK23(pIS284) and *S. lividans* TK23(pIS287) transformants are tested for the ability to bioconvert a high level (400 micrograms/ml) of daunorubicin to doxorubicin using the APM medium as described above. *S. lividans* TK23(pIS70) transformants can convert up to 11.5% of added daunorubicin to doxorubicin (Table 2). *S. lividans* TK23(pIS284) and *S. lividans* TK23(pIS287) transformants can convert up to 73.5% of added daunorubicin to doxorubicin (Table 2).

Table 2. Bioconversion of daunorubicin to doxorubicin by *S. lividans* strains.

Strain	Anthracycline (micrograms/ml)		
	DOX	DNR	13-dihydroDNR
S. lividans TK23(pIS70) *(control)*	46	250	70
S. lividans TK23(pIS284)	294	33	21
S. lividans TK23(pIS287)	288	24	35

Example 5

Doxorubicin production in the *S. peucetius* WMH1654 *dnrX* mutant transformed with plasmids containing the *doxA* daunorubicin C-14 hydroxylase gene together with different resistance genes: The pIS284 and pIS287 plasmids are introduced into *S. peucetius* WMH1654 *dnrX* mutant strain by protoplasts transformation with selection for thiostrepton resistance, according to the procedures described in the Materials and Methods section. The resulting *S. peucetius* transformants are fermented and the fermentation broths analyzed according to the method previously described. *S. peucetius* WMH1654(pIS284) produced up to 81 micrograms/ml of doxorubicin and up to 18 micrograms/ml of daunorubicin after a 120 hr fermentation (Table 3). *S.peucetius* WMH1654(pIS287) produced up to 92 micrograms/ml of doxorubicin and no detectable amount of daunorubicin (Table 3).

Table 3. Doxorubicin production by *S. peucetius* WMH1654 *dnrX* strains.

Strain	Anthracycline (micrograms/ml)		
	DOX	DNR	13-dihydroDNR
S. peucetius WMH1654	41	35	18
S. peucetius WMH1654(pIS284)	81	18	6
S. peucetius WMH1654(pIS287)	92	0	0

SEQ ID.1

1 GGATCCGCAC CGGGTACACG GCACGGGACC GCCCACCGCG CGGTGCGCGG

51 TGGGCGGTCC CGTGCCGGTC GCGGCCGGCG GATCAGCGCA GCCAGACGGG

101 CAGTTCGGTG AGCCGCGCCG TCTGGGCCCC CTTCCGGCAC CACCGCAACT

151 CGTCGTACGG CACGGCCAGT CGGGCCTCGG GGAACCTGCT GCGCAGTACG

201 CCGATCATCG TGCGCGACTC CAGCTGGGCG AGCTGCTCCC CGATGCAGTA

251 GTGCGGCCCG TCGCCGAAGG TGAGCCGCCG CCACGAGGGA CGGTCCGGGT

301 GGAAGGCGTG CGGGGCGTCG TGATGGCGGC CGTCGGTGTT GGTGCCCTCG

351 ATGTCCACCA GCACCGGCGC TCCGCGGGGC AGCCGGACGC CGCCGATGGT

401 CACCTCCGTG GCAGCGAACC TCCACAACGT GTAGGGCACC GGCGGGTGGT

451 AGCGCAGCGC CTCCTCCACG AACCGGGAGA CGGCGTCCTC GTCGGCATCC

501 GCCGCGAGGC GGCCCGCCAG GACCTCCGCG AGCAGGAAGC CCAGGAAGGA

551 GCCGGTGGTG TCGTGGCCGG CGAAGATGAG CCCGGTGATC ATGTAGACGA

601 GCTGGTCGTC GGAGACCGAG CCGAACTCGG CCTGCGCGCG CTCGTACAGC

651 ACGCGGGTCA TGGTCGGGGT GTCGTTCCGC CGGGCTGAGT GCACGGCTTC

701 GAGGAGCAGG CTCTCCAGGG CCGAGGTGTC CGGCACGCCC CCGGCAGGGT

751 CCGTGCCGTC ACCCCCGCCG CTCTGCGGGC CGCCGAGGCC GAGTGCCTTG

801 AGAACGCTGA CGGCCTCGCG GGCCATCGCC GGATCGGTGA CCGGCACACC

851 GAGCAGCTCG CAGATGACCA ACAGCGGGAA GTGGTACGCG AAGCCGCCGA

901 TCAGCTCGGC CGGTTTGCCC GACCGGCCGG AGGCGTCGGC GAGTTCGGTG

951 AGCAGCCGGC CGGCGATCGC GGCGATGCGA TCCGTCCGCT CGGCCAGCCG

1001 GCGCGGGTTG AACGCAGGTG CGTGGATGCG GCGCAGGCGC CGGTGGGCCT

1051 CGCCGTCCAC GGCGATGAGC GTGAACGGAC GCAGCTCCGG AACGGGGATG

1101 TCGAGACCGT CGTCCACCCC CCGCCAGGCG GCGGGGGCGA GGTCGGGGTC

1151 CTTCACGAAC CGGGGATCGG CCAGCACCTC GCGGGCGAGG GCGTCATCGG

1201 TGATGACCCA GGCGGGTCCG CCCGCGGGGG CGTTCACCTC GACGACCGGG

1251 CCCGCCTCCC GGAAGGCGTC GTGCACCTCG GGCTTGCGCT GCATGGTCAT

1301 CATGGGACAC GCGAACGGGT CGACGGCCAC CCGGGGCGCC TCGCCGCTCA

1351 CGAGGCACCG CCCGCCGCCG CGGGGTACCC CTCCCGCAGT TCGACCACCG

1401 AGAAGCCGGC CCCGTGCGGG TCGAGCAGGT CCGCCCGCCG CCCCCTGGGC

1451 GTGTCGGCGG GCTCGTTCTC GACGGAGCCG CCGAGTTCAA CGGCGCGCCG

1501 GACCGTCGCG TCGCAGTCGT GCACGGCGAA CAGCACGGCC CAGTGCGGCC

1551 GTACCGCGCC GGTGACGCCC AGCTCCTGGG TGCCGGCGAC CGGTGTGTCA

1601 CCGATGTGCC AGACCGGGTC GGTGACGCCC TTCAGTCCGG TGTCGGCCGG

1651 AGCCAGGCCG AGGGTCGCCG GGTAGAAGTC CCGGGCGGCC CCGATGCCGT

1701 CGGTCACCAG CTCGACCCAG CCGACCGAGC CGGGCACGCC CGTCACCTCC

1751 GCGCCCTCCA TGACTCCCTT GCGCCAGACC GCGAACGCGG CCCCGGCGGG

1801 GTCGGCGAAG ACCGCCATCC GGCCGAGGCC GAGGACGTCC ATCGGAGTCA

1851 TGATGACCTC GCCGCCCGCC GTCTCGACCC GCTTGGTCAG TGCGTCGGCG

1901 TCGTCGGTGG CGAAGTACAC GGTCCAGATG GCCGGCATGC CGTGCTGGTC

1951 GTTCCCGGGC CCGTACGGCC GGTGGTAGGG GGTGTCGATC TGGTGGCGGG

2001 CGACCGCGGC GACCAGCTTC CCGTCGGAGC TGAACGTCGT GTATCCCCCG

2051 GCGCCCGGGT CGCTGACCAC GGTGGCGGTC CAGCCGAACA GGCCGGTGTA

2101 GAAGTCGGCC GAGGCGGCGA CATCGGGCGA ACCGAGGTCG AACCATGCGG

2151 GGGCGCCGGG CGCGAACCTG GTCACGAATC GTTCCTTTCG ATGGATCGGC

2201 ACACGAGCGT CTGCGCTCGC GGATGAGACG GACATCTCGC GGATGAGACG

2251 GACATGCGGG CGGGGCGGGC CGCCGCCGTC AGTGCGCGGT GTCGCCGACG

2301 GCGGCCGCGC CGGCCTCCCA GAGCTTCGCC GCGAGGCCGG CGTCGGCGGT

2351 CGGGCCGCTC ACCGGGGACA GCCGCCGGTC GCTGTAGTAG CCGCCCGTGG

2401 TCAACTCCTC GGCCGGCGCG GACGCCAGCC ACACGAGGGT GTCGGCGCCC

2451 TTCGCCGCGG AGCGCAGGAA GGGGTTGAAC CGGAAGTAGG ACGAGGCGAC

2501 CGTGCCCCGT CCGATGCGGG TGCGGACCTC ACCGGGGTGA TAGCTGACCG

2551 CCAGCACGTC CGGCCAGCGC CTGGCGGCCT CCGCCGCGGT CATGATGTTG

2601 GCCTGTTTGG ACGTGCCGTA CGCCTGGCCG GCGCTGTAGC GGTGACGGTC

2651 GCCGTTGAGG TCGTCCGGGT CGATCCGGCC CTGGGTGTAC GCGTCGGACG

2701 AGGTGAGGAT CAGCCGCCCG CCCGCGAGCC GCTCCCGCAG CAGCCGTGCC

2751 AGCAGGAAGC CTGCGAGGTG ATTGACCTGG ATGGTGGCCT CGAACCCGTC

2801 CTGGGTCGTG GTGCGCGACC AGAACATGCC GCCGGCGTTG CTGGCCATGA

2851 CATCGATGCG CGGGTACCGG